The Influence of Dietary Energy Source on Microbial Diversity in the Equine Gastrointestinal Tract

Thesis

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Abstract

Horses are commonly fed diets that contain different sources of energy including starch, fiber and fats. These different energy sources may be digested and absorbed in differing segments of the gastrointestinal tract, thereby altering the gut microbiome. Six mature Miniature Horse geldings (7.5 ± 3.5 yr; 134.5 ± 39.5 kg) were used in a 6 x 6 Latin Square design to evaluate the effect of different dietary energy sources on the microbial diversity of the equine gastrointestinal tract. Diets consisted of mixed grass hay plus one of three energy supplements (oats, beet pulp, rice bran) at two levels (high: 75% supplement/25% hay or low: 40% supplement/60% hay). All diets were identical for digestible energy; however, dry matter intake (DMI) varied. On d 14 of each period, fecal samples were collected and DNA was extracted, pooled by treatment and subjected to PCR-DGGE with primers specific to 16S rRNA gene sequences to evaluate changes in bacterial diversity. PCR-DGGE images were analyzed with BioNumerics software to generate dendrogram comparisons based on the position and number of bands with further evaluation using Principal Coordinate Analysis (PCA). Although there were numerical differences in band counts in the microbial populations evaluated, these data could not be statistically analyzed due to the low sample number (n=1) per treatment group. However, PCA and dendrogram analyses revealed distinct clusters which indicate changes in the microbial profiles of horses fed different energy sources. Further research using species-specific primers is needed.

Introduction

Horses are commonly fed diets that contain different sources of energy including starch, fiber and fats to meet their caloric needs (Crandell et al. 1999). The use of concentrated energy sources in equine diets can lead to modifications of microflora and the biochemical characteristics of the large intestinal content (de Fombelle *et. al.,* 2001). In addition, these different energy sources may be digested and absorbed in differing segments of the gastrointestinal tract, thereby altering the gut microbiome (Keinzle, 1994).

The bacterial community within the equine digestive tract has been found to be extremely diverse and complex. The equine microbiome changes rapidly from birth until 56 d of age and even then adult horses have are reported to have a dynamic gastrointestinal microflora (Drogoul et al., 2001). Previous research has demonstrated that the type of feed can influence their gastrointestinal microflora. In mature horses, lower concentrations of lactic acid bacteria were observed when forage only diets were fed compared to concentrate diets (Willing et al., 2009). Sudden changes in a horse's diet have also been shown to alter both the diversity and abundance of microbial populations (Kern et al., 1973; de Fombelle et al., 2001; Dougal et al., 2001; Bailey et al., 2003; Berg et al., 2005). Increases in total anaerobic bacteria, *Lactobacilli* and *Streptococci* in the cecum and in the colon of horses were observed within 48 hrs of changing their diet from grass to hay (de Fombelle et al., 2001). However, lactate-utilizing and cellulolytic bacteria were not significantly modified after the ingestion of the new diet.

When horses consume large amounts of readily digestible carbohydrates, the starch that is unfermented from the small intestine will carry over into the hindgut of horses and potentially alter microbial populations (Willing et al., 2009). The objective of this study was to determine if

the dietary energy source, and level of inclusion, can influence microbial diversity in the equine hindgut.

Materials and Methods

Six mature Miniature Horse geldings (7.5 ± 3.5 yr; 134.5 ± 39.5 kg) were used in a 6x6 Latin Square design to evaluate the effect of different dietary energy sources on microbial diversity in the equine gastrointestinal tract. Horses were individually housed in box stalls and fed one of six diets for six 14 d periods. Diets consisted of mixed grass hay plus one of three energy supplements (oats, beet pulp, rice bran) at two levels (high: 75% supplement/25% hay or low: 40% supplement/60% hay). All diets were identical for digestible energy; however, dry matter intake (DMI) varied. Both forage and energy supplements were fed at 0700 h and 1900 h. Any orts were collected every 24 h prior to 0700 h feeding. Trace minerals were added to the ration once a day to meet daily nutrition requirements and water was provided *ad libitum*. Horses were turned out for 1 h/d in 12 m x 12 m dry lots to allow for maintenance of feed intake and allow adequate physical activity.

Within each period, the horses were given a 3 d period to transition to the new diet followed by a 7 d adaptation period. On d 14 of each period, fecal samples were collected and stored at -20º C until further analysis. One gram of feces from each horse was pooled by diet. DNA was extracted using a Repeated Bead Beating Plus Column RB++C method (Yu and Morrison, 2004). The subsequent DNA was purified using a Qiagen mini DNA kit (Qiagen Inc.; Valencia, CA). Quantification of DNA was determined using Quant-iT PicoGreen (Molecular Probes Inc.; Eugene, OR) kit with the following modifications: 50 µL of working solution was used instead of 200 µL, 2.5 µL of standards or DNA samples were used in each well rather than 10 µL. Purified DNA was subjected to electrophoresis on a 1% agarose gel at 20 v then 80 v for

1 hr. DNA was analyzed using PCR-DGGE. The primer used was specific to the V2-V3 region of 16S rDNA of all bacterial species (universal). The reaction mixture for PCR using universal the primer (HDA1, HDA2) (50 μL) contained 0.25 μL of each 100 μM primer and Taq polymerase, 50 ng of the DNA template, 5.00 μL of PCR reaction buffer (Invitrogen; Life Technologies Corp.; Eugene, OR), 1.02 μL of BSA, and 3.57 μL of 50 mM $MgCl_2$, and 0.408 μL 100 mM dNTP. Distilled water (Life Technologies Corp., Eugene, OR) was added to each reaction for a final total volume of 50 μL.

Microbial diversity from DGGE was evaluated with BioNumerics (Applied Maths NV; Saint-Martens Latem, Belgium). The microbial DNA bands in each lane were detected on the gel manually and using the automatic band search function. This allowed bands to be selected and deselected when necessary, thus allowing banding profiles to be determined. Gels were normalized using internal reference bands and external reference markers (100-bp ladder; Invitrogen Inc.; Carlsbad, CA). A 1.0 % position tolerance was set for band matching to help correct minimal migratory variation. The DGGE banding patterns were transformed into a binary (presence or absence of bands) correlation matrix in BioNumerics. Dendrograms were created based on the unweighted pair-group method with arithmetic average (UPGMA) and the Jaccard function. Similarity was represented with a similarity coefficient derived from the pairwise comparison of DGGE banding patterns between two samples. Principle coordinate analysis (PCA) was performed using a combination of BioNumerics and Microsoft Excel.

Results and Discussion

Fecal samples were pooled by diet thus creating a sample size of 1 per treatment group. Although numerical differences were observed in the microbial DNA bands between treatment groups; these band counts could not be statistically analyzed due to the low sample number (Figure 1).

The dendrograms generated by DGGE banding patterns revealed clustering by site of digestion in the microbial profiles of horses fed different dietary energy sources (Figure 2). PCA also revealed clustering by site of digestion (Figure 3). Samples from horses in the RBL, OH and OL treatment groups are clustered together and these dietary energy sources are digested in the foregut of horses (Keinzle, 1994). RBH is also digested in the foregut but is not included in the cluster with RBL, OH and OL. This may be due to the high level of inclusion and carry over of nutrients into the hind gut. Previous research has shown that unfermented starch from the small intestine will carry over into the hindgut of horses and alter microbial populations when horses consume a large quantity of readily digestible carbohydrates (Willing *et al.*, 2009).

BPH and RBH microbial profiles were clustered together even though the diets differed in energy source and site of digestion. While rice bran is digested in the foregut, beet pulp is digested in the hind gut of horses. Beet pulp is high in fiber and digested in the hind gut of horses. BPH was the only diet that resulted in orts during the study. The reduced intake when horses were fed BPH may have produced a similar result as the spillover of carbohydrates from RBH and account for the unexpected differences in profiles observed.

Of particular interest in this study was a specific microbial DNA band that was observed in all treatment groups except BPL. This unique difference between microbial profiles was also revealed in both the dendrogram and PCA. Horses fed BPL exhibited a distinct microbial

banding pattern compared to all other diets. Further research using species-specific primers is needed to better understand the differences in microbial profiles observed in this study.

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Appendix

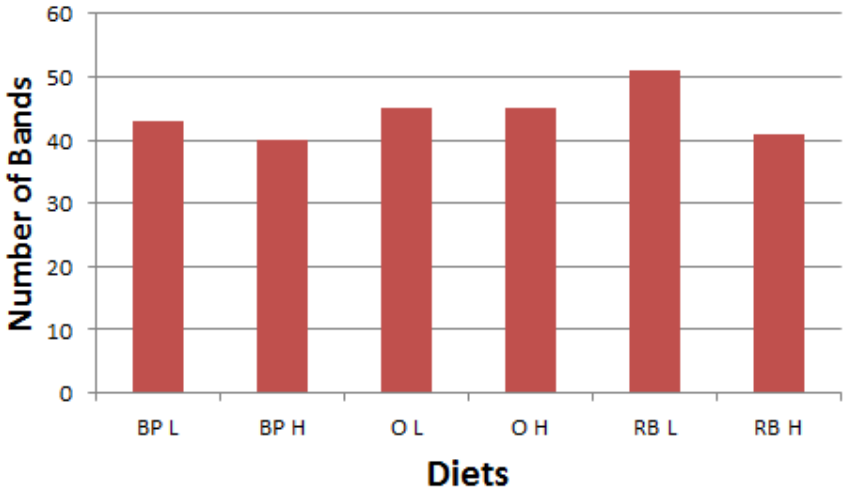

Figure 1. Influence of dietary energy source on hindgut microbial diversity in mature Miniature Horse geldings (n=6) as shown through band counts. Band counts were generated using primers specific to 16S rRNA sequences.

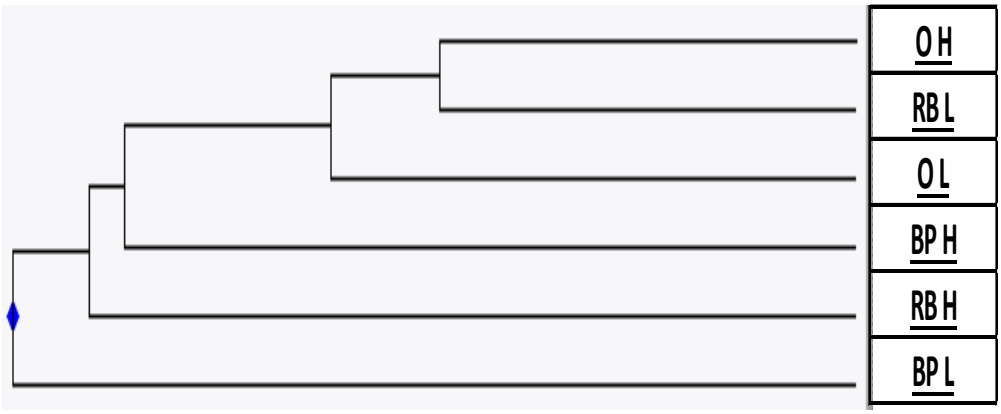

Figure 2. Relatedness of PCR-DGGE profiles representing total bacteria from pooled fecal samples from Miniature Horse geldings (n=6). Dendrograms were generated based on a distance matrix calculated by the Jaccard and unweighted pair-group method with arithmetic average (UPGMA) functions.

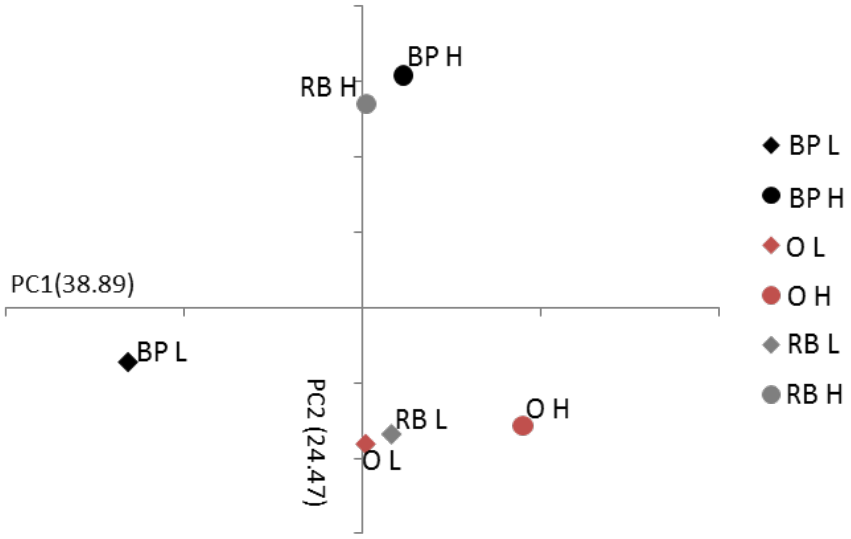

Figure 3. Relatedness of PCR-DGGE profiles representing total bacteria from pooled fecal samples from mature Miniature Horse geldings (n=6). PCA was generated from a binary similarity matrix to assess the similarity between treatments.